ated by a synaptic or neurosecretory route. Nor do the data demonstrate that the control of mucus release is the only effector role of these large neurons. But the control of mucus release by cells secreting 5-HT as a transmitter may be a more general phenomenon, for it has been reported that the direct application of 5-HT mediates the secretion of mucus in the mammalian stomach (*9*).

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Quantitation of RNA Tumor Viruses and Viruslike Particles in Human Milk by Hybridization to Polyadenylic Acid Sequences

Abstract. *RNA tumor viruses and viruslike particles from human milk are quantitated by hybridization of the polyadenylic acid regions in their 60S to 70S RNA to radioactive polyribouridylic acid of known specific activity. The length of the polyadenylic acid region in the 60S to 70S RNA of the human milk particle is identical to that of the known oncogenic RNA viruses.*

Particles that have several biochemical and biophysical characteristics unique to the RNA tumor viruses have been isolated from human milks (*1*). Utilization of the simultaneous detection technique (*2*) permitted the demonstration that these particles, with a density of 1.16 to 1.19 g/ml, contain an RNA-instructed DNA polymerase and a high-molecular-weight (HMW) RNA (60*S* to 70*S*), which serves as the template in an endogenous reverse transcriptase reaction (*3*). Numerous investigators have found that the 60*S* to 70*S* RNA of oncornaviruses contains polyadenylic acid [poly(A)] regions (*4–6*) that represent approximately 1.5 percent of the viral genome (*4, 5*). We report here that the 60*S* to 70*S* RNA from human milk particles also contains poly(A) stretches and that they are the same length, approximately 200 nucleotides long, as those found in the mouse mammary tumor virus (MMTV) RNA and in other RNA tumor viruses. Finally, we have used hybridization to poly(A) segments (*5*) as an assay for the detection and quantitation of these particles in human milks and for the quantitation of RNA tumor viruses in biological fluids.

The assay used in our study for the detection of poly(A) stretches in HMW-RNA is a modification of that of Gillespie *et al.* (*5*). Any viruses present were purified from biological fluids by velocity and equilibrium gradient centrifugations (legends to Figs. 1 and 2). RNA was extracted from particles with a density of 1.16 to 1.19 g/ml and then sized by means of glycerol velocity gradient centrifugation with the aid of appropriate external markers. Each fraction of the velocity gradient is tested for poly(A) regions by hybridization to [^{3}H]poly(U) (tritiated polyribouridylic acid). Ribonuclease and deoxyribonuclease treatment is used to ensure that the remaining acid-insoluble radioactivity in a given fraction is due to the hybridization of [^{3}H]poly(U) to the poly(A) region of an RNA molecule. Acid-insoluble radioactivity in the 60*S* to 70*S* region of the gradient constitutes a positive.

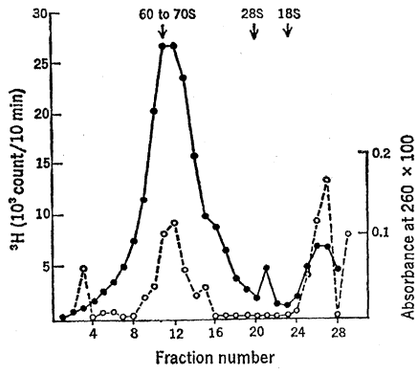

Fig. 1. Detection of polyriboadenylic acid regions in high-molecular-weight RNA from AMV. The AMV was purified by filtration and differential centrifugation (*14*). RNA was extracted from 2 mg of viral protein by treatment with sodium dodecyl sulfate (SDS), 1 percent; Pronase (self-digested), 750 μg/ml; and mercaptoethanol, 1 percent; for 30 minutes at 37°C in TNE buffer [0.1*M* NaCl, 0.01*M* tris(hydroxymethyl)aminomethane, *p*H 8.3, 0.015*M* EDTA]. The RNA solution was then mixed (1 : 1 : 1) with chloroform containing 1 percent isoamyl alcohol and phenol-cresol (7 : 1), *p*H 7.5, containing 8-hydroxyquinoline (0.37 g/100 ml) and shaken at 4°C for 10 minutes, and then centrifuged at 8000*g* for 10 minutes. This and all subsequent centrifugations were at 4°C. The aqueous phase was reextracted with the chloroform-phenol mixture, as described above, and then precipitated overnight at −20°C after addition of 0.4*M* NaCl and two volumes of cold ethanol. The RNA was then sedimented by centrifugation at 16,000*g* for 20 minutes; it was then dissolved in 0.5 ml of 0.003*M* EDTA and layered over a linear glycerol gradient (10 to 30 percent) in 0.01*M* tris (*p*H 8.3), 0.15*M* NaCl, 0.002*M* EDTA, and centrifuged at 40,000 rev/min for 3 hours (Spinco SW-41 rotor). The gradient was dripped and absorbancy (260 nm) was read for each 400 μl fraction. The poly(A) content of each fraction was then determined by hybridization to [^{3}H]poly(U). A 4-μl sample from each fraction was brought to 600 μl, the final solution containing 0.45*M* NaCl and 0.045*M* sodium citrate (3 × SSC), 0.003*M* tris (*p*H 7.0), 50 percent formamide, and 5000 count/min of [^{3}H]poly(U) (17 c/mmole or 9.3×10^3 count/min per picomole) and incubated at 36°C for 48 hours. Two milliliters of solution containing 0.01*M* tris, 0.01*M* $MgCl_2$, 0.5*M* NaCl, 10 μg of pancreatic ribonuclease A (Sigma) (boiled 10 minutes in 6 × SSC), and 40 μg of deoxyribonuclease I (Sigma) were added and the incubation was continued for 2 hours at 30°C; the mixture was then placed at 4°C for 15 minutes and precipitated with 0.5 ml of 100 percent trichloroacetic acid containing 0.2 percent uridine (P-L Biochemicals). After incubation for 30 minutes at 4°C, acid-precipitable radioactivity was determined by collection on membrane filters, followed by washing with 10 percent trichloroacetic acid containing 0.02 percent uridine. Closed circles, ^{3}H, 10^3 count/10 min; open circles, absorbancy at 260 nm × 100.

This technique has been successfully used here to detect and quantitate both type B and type C viruses in milk, plasma, or tissue culture fluids. An example of such an assay with avian myeloblastosis virus (AMV) from chicken plasma is illustrated in Fig. 1. RNA from particles was extracted and sedimented on a glycerol velocity gradient (10 to 30 percent) by centrifugation at 40,000 rev/min for 3 hours at 4°C; 28*S* and 18*S* RNA from NC-37 cells were used as external markers. As an added control the absorbancy at 260 nm of each fraction was taken to determine the region of HMW-RNA and a peak of 0.11 absorbancy unit appeared in the 60*S* to 70*S* region. One-hundredth of each fraction was then assayed for poly(A) stretches. As seen in Fig. 1, a distinct peak of [^{3}H]poly(U) (2600 count/min) is found in the HMW-RNA region, corresponding to the 0.001 absorbancy unit expected. It is evident, therefore, that the viral RNA can be detected by this assay where the amount of RNA present is too small to detect by absorbancy or where radioactive RNA samples are difficult to prepare, for example, with human biological fluids such as milk or plasma.

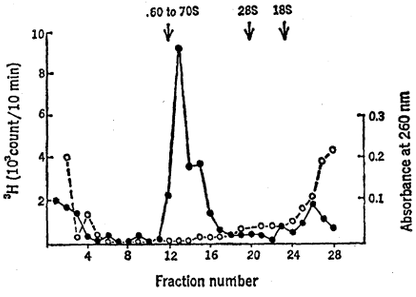

Fig. 2. Detection of polyriboadenylic acid regions in high-molecular-weight RNA from human milk particles. Human milk (17 ml) was treated with an equal volume of 0.15*M* EDTA (*p*H 7.5) and centrifuged at 3000*g* for 15 minutes; the clear "milk plasma" interface was then placed on an 8-ml column of 20 percent glycerol over a 6-ml pad of 100 percent glycerol in a Spinco SW-27 centrifuge tube and centrifuged at 98,000*g* for 60 minutes. The material on top of the 100 percent pad was skimmed off and layered on top of a linear sucrose (25 to 50 percent) equilibrium gradient. After centrifugation at 98,000*g* for 3 hours, the material from the 1.16 to 1.19 g/ml density region of the gradient was pooled, and the RNA was extracted and sized as described in Fig. 1. The absorbancy (260 nm) was determined and 200 μl of each fraction was assayed for poly(A) content, as described in Fig. 1, with the exception that 2500 count/min of [^{3}H]poly(U) was used per fraction. The [^{3}H]poly(U) was synthesized by incubation of [^{3}H]UDP (180 μg/ml) (Schwarz-Mann; 17 c/mmole) in 0.1*M* tris (*p*H 8.1), 0.003*M* EDTA, and polynucleotide phosphorylase (200 μg/ml) (Worthington) for 20 hours at 30°C. Then 0.5 percent SDS, 0.1 volume of isoamyl alcohol, and 1 volume of chloroform were added, mixed for 2 minutes (Vortex), and centrifuged at 16,000*g* for 10 minutes. The aqueous phase was precipitated by the addition of 0.2*M* NaCl and 2 volumes of cold ethanol, stored at −20°C overnight, and centrifuged at 16,000*g* for 15 minutes. The pellet was dissolved in 0.2*M* NaCl and reprecipitated with ethanol overnight. The preparation was then centrifuged at 16,000*g* for 15 minutes, and the resulting pellet was dissolved in 0.1 ml of water and 0.1 ml of ethanol per millicurie of [^{3}H]UDP used, and stored at −20°C. Closed circles, ^{3}H (10^3 count/10 min); open circles, absorbancy at 260 nm.

It is also possible to quantitate the number of virions in a given preparation by means of this assay. Previous studies have demonstrated that (i) RNA tumor viruses contain a HMW-RNA of 1.2×10^7 daltons or 2×10^{-11} μg (*7*); (ii) poly(A) regions represent approximately 1.5 percent of the HMW-RNA genome (*4*, *5*); and (iii) there are 3.3×10^3 pmole of RNA per microgram of RNA. From this it is evident that there are 9.9×10^{-10} pmole of adenosine monophosphate in the poly(A) stretches of each virion. The number of virions per preparation can now be determined:

No. virions =
the total counts per minute in the 60*S* to 70*S* RNA region × (9.9×10^{-10} × the specific activity of [^{3}H]poly(U) in counts per minute per picomole)$^{-1}$.

The accuracy of this calculation depends on the validity of the molecular weight of the RNA and of the estimated proportion of poly(A) segments. The number of virions of AMV used in this assay (Fig. 1) can be calculated at 1.33×10^9 from the above-mentioned formula. This is in agreement with the value obtained from the measurement of the absorbancy at 260 nm.

We have successfully used the poly-(A) hybridization technique to detect and quantitate Rauscher murine leukemia virus in mouse plasma, mammary tumor virus in mouse milk, Mason-Pfizer monkey virus in NC-37 cell culture supernatants, and particles in human milk. As described in the legend to Fig. 2, human milk preparations were first centrifuged at 3000*g* to remove any free nuclei. The viruslike particles were then separated from free nucleic acids and light membrane fragments by a velocity centrifugation through 20 percent glycerol and further purified by sucrose equilibrium gradient centrifugation. The RNA from particles at densities of 1.16 to 1.19 g/ml was extracted, sedimented, and assayed for poly(A) regions. The distinct peak of radioactivity observed (Fig. 2) indicates a 60*S* to 70*S* RNA molecule containing poly(A) regions. However, quantities with absorbancy at 260 nm were not observed in the HMW-RNA region. From the formula above, this milk contained 3.2×10^8 particles or 1.9×10^7 particles per milliliter. This would correspond to 7.8×10^{-5} absorbancy (260 nm) unit of HMW-RNA, an amount too low to detect spectrophotometrically. In several milk samples, some poly(A) stretches were also observed in the 4*S* to 28*S* region of the gradient; this is not surprising and is most probably due to contaminating cellular RNA that contain these sequences (*8*). To further elucidate the nature of this 60*S* to 70*S* RNA molecule, the size of the poly(A) region was determined by acrylamide gel electrophoresis (*5*). The size of the poly(A) region, that is, 200 nucleotides, is virtually identical to that found in the mouse mammary tumor virus (Fig. 3) and to that previously reported (*5*) for murine and feline leukemia viruses, Mason-Pfizer monkey virus,

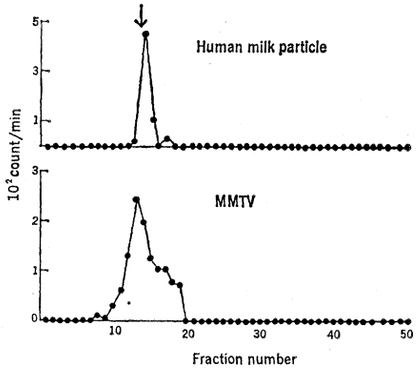

Fig. 3. Polyacrylamide gel electrophoresis of poly(U)·poly(A) hybrids. A 400-μl portion from the HMW-RNA region shown in Fig. 2 was hybridized to 10,000 count/min of [^{3}H]poly(U) in a standard hybridization mixture for 48 hours and treated with nuclease for 2 hours in a total volume of 3.8 ml. After addition of SDS to a final concentration of 0.5 percent, the solution was extracted with 1 volume of chloroform and 0.1 volume of isoamyl alcohol, and the aqueous phase was precipitated with 2 volumes of ethanol at −20°C. The precipitate was collected by centrifugation at 13,700*g* for 2 hours, and gel electrophoresis was carried out as described (*5*), but on 7.5 percent acrylamide gel.

and avian myeloblastosis virus (Fig. 3, arrow).

We have tested 37 samples of different human milks for a 60*S* to 70*S* RNA containing poly(A) sequences. Five, or 14 percent, were positive, ranging in particle counts from 1.0×10^6 to 4.0×10^7 particles per milliliter. A distinct peak at 35*S* was observed in a sixth sample. The procedures for particle purification were designed to eliminate free messenger RNA's and nuclei containing heterodisperse nuclear RNA, both of which contain poly(A). The fact that 86 percent of the milks tested were negative is an indication that the 14 percent positives are not due to normal RNA species of human lactating mammary tissue that are usually found in milk. Further, a diagnostic distinction can be made between the poly(A) stretches in the RNA of the human milk particle and those found in cellular messages. Gillespie *et al.* (*8a*) have shown by electrophoretic analysis that the poly(A) regions in cellular messages are extremely heterogeneous in size. In contrast, the poly(A) stretches in the 60*S* to 70*S* RNA of the human milk particle cluster sharply at a size range corresponding to 200 nucleotides (Fig. 3), a finding in agreement with that observed in the RNA tumor viruses (*5*). The RNA's of other mammalian viruses, such as poliovirus and eastern equine encephalitis virus, also contain poly(A) regions (*9*). However, neither the size of the RNA nor the length of the poly(A) region reported for these viruses corresponds to the results obtained here.

Numerous investigators (*10*), employing electron microscopic techniques, have reported the occasional presence of particles resembling RNA tumor viruses in human milk. These observations, however, depend not only on adequate numbers of particles, but particles that are morphologically intact. It has been shown (*11*) that human milks contain substances that destroy the morphological integrity of known RNA tumor viruses that are added. Viral particles, however, with outer membranes damaged or visually obscured by cellular debris, would still be detected by the poly(A) hybridization technique. The poly(A) assay depends only on the presence of an intact 70*S* RNA; thus, if only viral cores were present, a positive response would still be obtained (*12*). The poly(A) assay may also be used to detect defective mutants, such as RSV_α described by Hanafusa and Hanafusa (*13*), that do not contain reverse transcriptase. Finally, the poly(A) hybridization technique provides a useful method for quantitation of known and putative RNA tumor viruses.

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Soluble Membrane Antigens of Lip and Cervical Carcinomas: Reactivity with Antibody for Herpesvirus Nonvirion Antigens

Abstract. *With the use of antibody for herpesvirus nonvirion antigens (not structural components of the virus) complement fixing reactivity has been shown for soluble membrane antigens separated from lip and cervical carcinomas but not for similar extracts from normal vaginal tissue or intestinal carcinoma. Neither the serum obtained from the guinea pig before hyperimmunization with the herpesvirus nonvirion antigen nor the antiserum of guinea pigs immunized with comparable uninfected cell extracts reacted with these tumor soluble membrane antigens. Since the above soluble membrane antigens could be specific markers for the presence of virus genome within the tumor cells, the findings could support an etiological role of herpesvirus in selected human malignancies.*

Antiserums to virus-specific, labile, nonvirion antigens in cells infected with herpes simplex virus (HSV) can be prepared by special absorption procedures (*1*). Specific, labile HSV-related soluble membrane antigens can be separated from invasive cervical tumor cells (*2*).

We now report that soluble cell membrane antigens extracted from carcinoma of the lip and from carcinoma of the cervix would react with antibody to HSV nonvirion antigens. An association of HSV subtype 2 with cervical cancer and of HSV subtype 1 with lip cancer has been suspected (*3*).

Normal vaginal tissue and invasive cervical, lip, and intestinal carcinomas were processed for preparation of soluble fractions from viable cell membranes (*2*). A 10 percent suspension of HEp_2 cells infected with the HSV type 2 (SAV strain) was used for the preparation of the virion and nonvirion antigens as well as for the antiserum to the partially purified HSV 2 (*2*). Virion and nonvirion antigens were extracted from HEp_2 cells and guinea pig kidney cells infected with the Schooler strain of HSV type 1; antiserums to these were prepared (*1*, *4*). The extracts of